# DEFINING MOLECULAR MECHANISM OF EGRESS IN CRYPTOSPORIDIUM

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## List of Abbreviations

| | |
|---|---|
| UTMB | University of Texas Medical Branch |
| GSBS | Graduate School of Biomedical Sciences |
| TDC | Thesis and Dissertation Coordinator |
| ART | Antiretroviral Therapy |
| PCR | Polymerase Chain Reaction |
| DALYs | disability-adjusted life years |
| TKGFR | tyrosine kinase growth factor receptor |
| RNAi | ribonucleic acid interference |
| RNA | ribonucleic acid |
| RISC | RNA-induced silencing complex |
| Ago | Argonaute |
| CDPK | calcium-dependent protein kinase |
| SCID | Severe combined immunodeficiency |
| PKG | cGMP-dependent protein kinase G |
| SUB1 | subtilisin-like serine protease 1 |
| HCT | Human ileocecal adenocarcinoma |
| PTR | protein transfection reagent |

| | |
|---|---|
| RT | room temperature |
| CFSE | Carboxyfluorescein succinimidyl ester |
| WT | wild-type |
| PBS | Phosphate buffered saline |
| DAPI | 4′,6-diamidino-2-phenylindole |
| CAM | Ca2+/Calmodulin |
| SERA | Serine-repeat antigen protein |
| LDH | lactate dehydrogenase |
| *C. parvum* | *Cryptosporidium parvum* |
| *T. gondii* | *Toxoplasma gondii* |
| *P. falciparum* | *Plasmodium falciparum* |

# DEFINING MOLECULAR MECHANISM OF EGRESS IN CRYPTOSPORIDIUM

## CHAPTER 1: INTRODUCTION TO CRYPTOSPORIDIUM

### *Cryptosporidium*: discovery and history

In 1907, Tyzzer first observed *Cryptosporidium* in the peptic mucosa of mice[3]. He described in detail asexual and sexual stages of the life cycle as well as an "organ of attachment", all with just light microscopy. In 1910, Tyzzer named the organism *Cryptosporidium*, noting common characteristics with coccidians[4]. The association of *Cryptosporidium* as a causative agent of disease was not suggested until the 1950s when oocysts were found in symptomatic turkeys[5]. In 1971, *Cryptosporidium* infection was also identified in bovines with diarrhea[6]. The first report of human cryptosporidiosis was pubished1976, in a child the age of three [1,4,7].

In the early 1980s, *Cryptosporidium* oocysts were found associated with diarrhea cases in the newly recognized AIDS epidemic. One study described cryptosporidiosis outbreaks in AIDS patients as, "…severe, unremitting and refractory to all therapy."[8]. By the mid-1980s, *Cryptosporidium* was regarded as a major cause of chronic diarrhea in HIV/AIDS patients[9]. Today, even with the introduction of antiretroviral therapy (ART), diarrhea remains a major complication of HIV/AIDS patients[10-12]. A recent study analyzed global outbreaks and found that in the developing world, approximately 90% of HIV/AIDS patients suffer from diarrhea and 30-60% in the developed world[13]. *Cryptosporidium* has been found to be one of the main pathogens that causes diarrhea in HIV/AIDS patients, with higher incidence in areas with poor or low sanitation facilities[14].

By the mid-1980s, waterborne outbreaks of *Cryptosporidium* were being recognized[15]. In 1984, *Cryptosporidium*-associated outbreaks were linked to drinking water[16]. A major outbreak took place in 1987 in Georgia that affected approximately 13,000 people[17]. It was found that water purification facilities were contaminated with *Cryptosporidium* oocysts, however the water had passed all current tests[15]. In 1993, a major outbreak took place in Milwaukee, WI affecting an estimated 403,000 people[18]. By the end of the 1980s, the association of pediatric disease and *Cryptosporidium* was becoming increasingly clear[19]. Several reports found *Cryptosporidium* oocysts in the stools of nearly one-third of hospitalized children with diarrhea[20,21]. Within a decade, *Cryptosporidium* was identified in children in all continents[1]. Around this time, the association of cryptosporidiosis and malnutrition was established, as well as the increased risk of mortality in severe infections[22-24].

## Global Burden of Cryptosporidiosis

In the past, a substantial proportion of cryptosporidiosis cases were not diagnosed due to the requirement for specialized tests and poor sensitivity of these tests. Clinicians often do not pursue the diagnoses, especially for self-limiting infections. Acid-fast staining of stool specimens was frequently used to diagnose cases of cryptosporidiosis. This method is inexpensive however it requires laboratory skills. Staining with microscopy is less than 70% sensitive[25]. When compared to molecular methods, up to 50% of cases are missed[9]. Enzyme immunoassays are another common method to diagnose illness, however there is a wide variability in sensitivity (~70-100%) depending on the method employed[25-28]. False positive tests are also an issue. Polymerase chain reaction (PCR) is the preferred method to diagnosis a *Cryptosporidium* infection. PCR has high sensitivity[29] and can even help researchers to differentiate between

different species of *Cryptosporidium*[30]. However, the cost of high-sensitivity methods for diagnosis is difficult for communities in resource poor settings to adopt.

The global burden in children is becoming increasingly clear. Nearly one-fifth of children worldwide are affected by gastrointestinal infections[31,32]. Introduction of oral rehydration therapies decreased the mortality related to acute infection, but growth impairments from persistent and recurring infections are being increasingly recognized[33-38]. Results from studies using sensitive techniques have further revealed that approximately 15-25% of children in resource-poor countries with diarrhea are positive for *Cryptosporidium*[39-43]. Several recent multinational studies have examined the pathogens causing diarrhea in children. In 2013, a three-year prospective case-control study sought to estimate the cause of moderate-to-severe diarrhea in children under 5 years of age in 7 centers in Sub-Saharan Africa and South Asia[44]. *Cryptosporidium* was the second most common pathogen associated with diarrhea in the first year of life, and the third most common in the second year of life[44]. This study found that most diarrheal-associated deaths occurred in the first two years of life (88%) and *Cryptosporidium* was significantly associated with mortality. This data later was extrapolated to estimate the frequency of *Cryptosporidium*-associated diarrhea in children throughout Sub-Saharan Africa and South Asia[45]. It was estimated that over 7.6 million children are affected with cryptosporidiosis annually with over 200,000 childhood deaths[45]. This data was later confirmed by another study that utilized the Global Surveillance Network to analyze stools from children under the age of five in 16 different countries[46].

In 2015, a multi-center birth cohort study (MAL-ED) aimed to estimate the causative pathogens for diarrhea and malnutrition. The study followed birth cohorts of young children from eight sights in South America, Africa and Asia[32]. *Cryptosporidium* was among the top five pathogens detected in stool collections from children in both the first and second year of life. Similarly, *Cryptosporidium* had a higher prevalence in children with moderate/severe diarrhea as

well as prolonged disease[32]. *Cryptosporidium* was associated with dehydration and infection risk was highest in overcrowded living situations[45]. Other birth cohorts from Asia have noted that 65-100% of children are infected by *Cryptosporidium* by age 2, but many of these infections are not associated with diarrhea[45,47,48].

A 2018 meta analyses attempted to determine the global burden of disease due to *Cryptosporidium* in young children[49]. Diarrhea due to *Cryptosporidium* was estimated to cause over 4.2 million disability-adjusted life years (DALYs) lost annually[50]. Additionally, each cryptosporidial episode resulted in decreased growth with a decrease of height-for-age Z score (HAZ)[51]. This meta-analysis estimated an additional 7.85 million DALYs from *Cryptosporidium*-associated nutritional defects[49]. Recent advances have also associated severe and persistent gastrointestinal infections to malnutrition and growth impairments in young children[22-24,52,53].

## Treatments: existing and development efforts

### *Cryptosporidium*: Parasite and Research

*Cryptosporidium* is a single-cell eukaryotic protozoan. It belongs to the phylum Apicomplexa, which also include *Plasmodium* and *Toxoplasma*. As of 2013, there have been 26 species identified that infect a variety of hosts from reptiles to birds to mammals, including humans. Two particular species, *C. parvum* and *C. hominis*, cause the majority of human infections, although infections with other subspecies have been reported as well[1]. *C. parvum* was initially believed to be the only species to infect mammals; however molecular studies have shown that *C. hominis* and other species can cause human infection[54].

*Cryptosporidium* is an obligate intracellular pathogen and usually completes its lifecycle in a single host. The life cycle includes asexual (merogony) and sexual (sporogony) reproductive

cycles (Fig 1)[1,55,56]. The life cycle begins with ingestion of infectious oocysts through contaminated food, water, or person-to-person contact. The oocyst infectious dose varies by subtype and host but has been reported to be less than 10 oocysts for some strains[57,58]. When oocysts reach the stomach of the host, they are exposed to low pH, proteases, peptidases, bile and body temperature which cause excystation of sporozoites [56,59]. Sporozoites are elongated invasive stages with an apical complex that contain specialized secretory organelles that aid in invasion. Sporozoites glide along the surface of intestinal epithelial cells, binding to surface receptors. Ligands in *Cryptosporidium* include; Cp30, galactose-N-acetylgalactosamine lectin, Cp23, gp900, Cp40/15, Cp47, the 1300-kDa circumsporozoite-like antigen, and the thrombospondin-related adhesive protein[1,55,56,59]. Parasite attachment causes a depression on the host cell surface, which causes host membrane protrusion around the parasite attachment site. This protrusion becomes intensified with host actin polymerization triggered by host CDC42, phosphatidylinositol 3 kinase (PI3K), and tyrosine kinase growth factor receptor (TKGFR)[1,55,60-62]. The host membrane engulfs the parasite in the parasitophorous vacuole which separates parasite from host cytoplasm[63]. An electron-dense band forms at the point of contact between parasite and host cytoplasm. This dense includes an ATP-binding cassette and transporters involved with acquisition of nutrients from the host[55,64]. At this point, the parasite undergoes asexual replication where type I meronts are formed and then mature into merozoites. Merozoites are motile and escape the host cell to reinvade uninfected

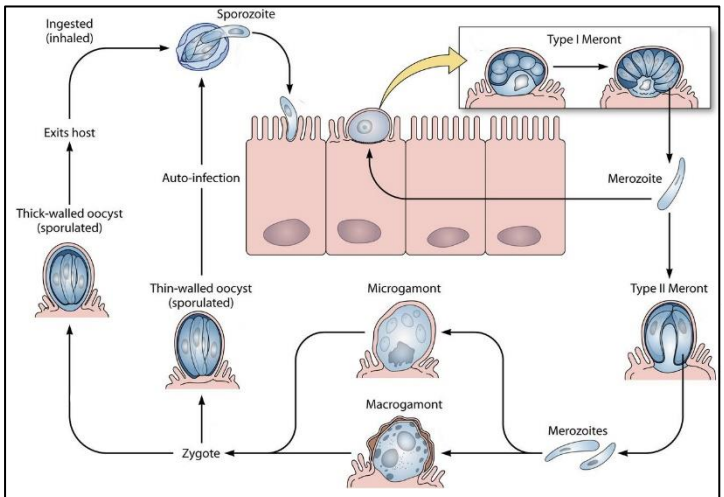


**Figure 1**. *Cryptosporidium* life cycle. Lifecycle of *Cryptosporidium* begins with ingestion of oocysts. In the small intestine, sporozoites hatch from oocysts and invade intestinal epithelial cells. Asexual replication produces merozoites that egress from host cells. Liberated parasites will invade an intact host cell and either repeat asexual replication or undergo sexual replication to produce zygotes that mature to oocysts and either autoinfect the host or exit the host in feces. (adapted from White, AC 2010)[1].

intestinal epithelial cells. Merozoites then either repeat asexual replication or progress into sexual differentiation. During sporogony, merozoites differentiate into micro- and macrogamonts. Microgametes release microgametes, which infect the cells harboring macrogamonts. The microgamete and macrogamont fuse to form the zygote which differentiates into one of two types of oocysts. Thin-walled oocysts remain in the host and begin an auto-infection process, while thick-walled oocysts exit the host and are released into the environment[1].

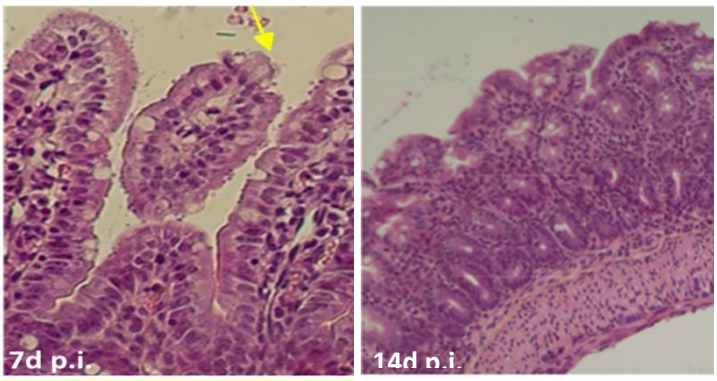


**Figure 2**. Pathogenesis of *Cryptosporidium* infection. Immunohistological images of ileum infected with *Cryptosporidium parvum* at 7 and 14 days post-infection. Images show villous atrophy and crypt hyperplasia as infection progresses. The arrows show the localization of parasite invasion. (adapted from Bai *et al*, 2018).

## Pathology & Pathogenesis

*Cryptosporidium* is localized to the microvillus layer of the distal small intestine and proximal colon in immunocompetent patients. When the immune response is deficient, infection can spread throughout the gastrointestinal tract, as well to extra-intestinal locations, such as the biliary and respiratory tracts[65,66]. Infection affects the structural integrity of villi and crypts in the intestine[67,68]. Pathology in severe cases is characterized by villous atrophy, crypt hyperplasia, infiltration of immune cells, and extra intestinal manifestations[66,69-72]

Symptoms include a watery diarrhea, dehydration, fever, nausea, among others. In normal hosts, symptoms last approximately 5-14 days. Children with persistent infection will also experience malabsorption which can lead to malnutrition and defects in growth and development[1]. Diarrhea is caused by a decrease in intestinal absorption and an increase in secretion[70,73]. The loss of villous surface leads to a decrease in sodium-pumps and fluid malabsorption[70,74]. Infection of intestinal epithelial cells will also lead to host cell death, which further affects the integrity of the

intestinal barrier[75,76]. Increased permeability in intestinal barriers can also be contributed by decreased absorption as well as influx of solutes in the gut. Substance P, a neuromodulator neuropeptide, has been correlated with severity of diarrhea[77]. Substance P mediated increased intestinal permeability and chloride secretion in animal models[78,79].

In the host cell, *Cryptosporidium* activates and secretes components which lead to alteration of cell function. A recent study demonstrated that *Cryptosporidium* infection negatively affects a miRNA family (let-7i) which regulates the expression of TLR4 in epithelial cells[82]. This intervention leads to a decrease release of anti-microbial peptides, such as β-defensins, which have demonstrated protective response to invasion by *Cryptosporidium*[61,83,84]. The parasites secrete non-coding RNAs in the host cytoplasm which has been found to lead to activation and secretion of pro-inflammatory cytokines[85,86]. In biopsy samples from infected humans, IL-8, IL-6, CXCL2 have been found; as well as tumor necrosis factor-a (TNF-a), interleukin 1b (IL-1b), and lactoferrin[87-91]. Infected epithelial cells will secrete IL-8 and CXCL10, which are potent pro-inflammatory chemokines[91-94].

Immune Response in Cryptosporidiosis

Innate and adaptive immune responses are critical for control and clearance of *Cryptosporidium*[9,91,95-97]. The innate response controls the intensity of an acute infection[98]. Once infected, intestinal epithelial cells release chemokines that attract mononuclear phagocytes (DCs, macrophages, monocytes) to the site of infection. Infected cells will secrete TNF-α and IL-1β, which increase intestinal permeability[99]. Macrophages and dendritic cells will secrete IL-12, which function to recruit lymphocytes as well as initiate the production of IFN-γ[100]. Dendritic cells activate the adaptive response and are essential for resolution of infection. When DCs are depleted in a mouse model, there is an associated increase of susceptibility to infection[101].

Macrophages and monocytes also produce IL-18 in response to infection, which in conjunction with IL-12, IL-15, and TNF-α will lead to the activation of natural killer cells (NK)[55,102]. Natural killer cells lyse infected cells through the release of cytotoxic granules[97]. Furthermore, NK cells are an important source of IFN-γ[101-104]. IFN-γ has been shown to play a key role in inhibiting *Cryptosporidium* invasion and intracellular development[105,106]. This has been demonstrated in SCID/IFN-γ KO mice that harbor a heavier infection when compared to SCID mice[107]. Similarly, IL-12 KO mice are susceptible to infection, which is a precursor that leads to IFN-γ production[108]. Human volunteers challenged with *Cryptosporidium* will express IFN-γ in the intestinal mucosa, although this expression is linked with a previous exposure[109].

Additionally, infected epithelial cells also produce antimicrobial peptides which kill extracellular parasites or induce apoptosis in infected cells[97]. Infection activates NF-κB signaling through stimulation of TLR2 and TLR4. This signaling cascade will lead to human β-defensin 2 production to aid in parasite clearance[61,110].

The adaptive response in cryptosporidiosis is required for the resolution of disease and prevention of reinfection[55,98]. CD4+ T cells are essential for clearance of parasite during the acute phase of infection[55]. Furthermore, disease is more frequent and severe in patients that are immunocompromised and exhibit low CD4+ T cell counts[105,111-113]. In patients that are HIV+, cryptosporidiosis is self-limited with T cell counts higher than 150/µL and become detrimental in those with counts less than 50/µL[114-118]. With the use of highly active antiretroviral treatment (HAART), there is an increase of CD4+ T cells followed by a decrease of symptoms[119]. Similarly, murine studies demonstrated increased severity of disease with depletion of CD4+ T cells (CD4 knockout, nude, or SCID mice)[95,96]. IFN-γ is a key mediator of the adaptive response to infection of *Cryptosporidium*. This is observed in *in vitro* studies where treatment of IFN-γ directly interacts

with intestinal epithelial cells to prevent invasion of *C. parvum*[106]. Furthermore, depletion of IFN-γ further exacerbates disease, even beyond what is observed from CD4+ T cell depletion[120]. In patients that have recovered from infection, lymphocytes will continue to produce IFN-γ after *C. parvum* antigen stimulation, regardless of HIV status[121-123]. However, in HIV+ patients, the levels of IFN-γ were low, which indicates that other factors are necessary in controlling infection. As mentioned, previously, macrophages and dendritic cells will produce IL-12 and IFN-γ in response to infection. These proinflammatory cytokines will induce differentiation of naïve T cells into Th1 cells which produce IFN-γ to induce a cell-mediated immune response. Th1 cells are essential in that they directly inhibit *Cryptosporidium* development.

Other factors also play a role in controlling cryptosporidiosis. Th17 cells, a subset of CD4+ T cells, are the first to differentiate in response to signaling from antigen-presenting cells (APCs)[97]. Dendritic cells will secrete IL-6 and TGF-β, and IL-23 to activate Th17 cells. CD8+ T cells have shown little direct effect on disease; however, they produce IFN-γ in response to infection which can clear parasites from infected cell *in vitro*[121,124,125]. Similarly, CD8+ T cells will also lyse infected cells by secreting cytotoxic granules. Studies in murine models that lack CD8+ T cell activity show no effect on resolution of infection compared to controls[126].

Th2 cells are less important in control of the parasite. However, the Th2 cytokine IL-4 may assist in the resolution of cryptosporidiosis[127]. Release of IL-4 will induce the differentiation of Th2 cells, which in turn will secrete IL-4, IL-10, IL-5, and IgG1, facilitating a humoral response[97]. However, the role of the humoral response in *Cryptosporidium* infection is unclear[91]. While Cryptosporidium-specific antibodies (IgM, IgA, and IgG) are found in serum, they are insufficient to prevent and control infection[128]. In mice, treatment with *Cryptosporidium*-specific antibodies demonstrated clearance of infection[129]. Despite this, there is some data that suggests antibodies play a more supportive role. Studies that utilize bovine colostrum have found that passive immunity confers protection in newborn calves[55].

In the host cell, *Cryptosporidium* induce activation of NFκB which will lead to host cell apoptosis[98]. Initially, the parasite will inhibit apoptosis in host cells to facilitate parasite replication and nutrient uptake. *Cryptosporidium* will activate NF-κB to initiate anti-apoptosis signalling[75]. Furthermore, the parasite will boost anti-apoptotic signals through Bcl-2 and survivin activation[131,132]. *Cryptosporidium* will also induce the secretion of osteoprotegerin, which serves as a decoy receptor to inhibit apoptosis[133]. After development, the parasite will facilitate apoptosis in host cells to facilitate egress. Apoptosis is mediated through activation of Fas and Fas-L molecule in both infected cells and surrounding uninfected cells[81].

## Treatment options for Cryptosporidiosis

Current chemotherapeutic options for cryptosporidiosis are not optimal. However, several drugs have shown anti-cryptosporidial activity[9,134]. In immunocompromised patients, treatment is most effective with the restoration of cellular immune function, e.g. with the use of antiretroviral therapy in AIDS patients[134,135]. Macrolide antibiotics have demonstrated some anti-cryptosporidial activity[134,136]. Studies looking at the activity of spiramycin in cryptosporidiosis found no significant decrease in disease incidence compared to controls[136,137]. Reports have demonstrated some activity of azithromycin in animal studies as well as HIV and cancer patients. However, when compared to placebo controls, no reduction in oocyst shedding was found[136]. Currently, Nitazoxanide is the only FDA-approved therapeutic for cryptosporidiosis and studies have observed a benefit in patients who are immunocompetent[138-140]. In 2002, a study investigated the effect of Nitazoxanide in Zambian children with *Cryptosporidium*-associated diarrhea. This study saw a 56% resolution of symptoms versus 23% in the placebo group[140] Efficacy studies on Nitazoxanide demonstrated resolution of symptoms in Egyptian adults and children with *Cryptosporidium*-positive stools. Results showed 80% of treated patients, versus 41% in

placebo[138]. In HIV/AIDS patients with low CD4 cell counts, on the other hand, Nitazoxanide was not effective without accompanying antiretroviral therapy[140-142].

Cryptosporidiosis Vaccination

There are no effective vaccines for prevention or treatment of cryptosporidiosis. However, there are several key observations that indicate vaccine development is feasible[9,143]. For instance, there is an association of increased susceptibility and severity of disease with the decline of cellular immunity in HIV patients[9]. Similarly, adults who live in areas with high burden of illness exhibit partial immunity to reinfection. Prior infection or evidence of prior infection increases the infectious dose in human challenge studies [57,58].

Several surface proteins in *Cryptosporidium* sporozoites are highly immunogenic. This immunogenicity has been the foundation for development of DNA and subunit vaccines. DNA vaccines encode a surface protein (preferably a highly-immunogenic protein) that will lead to a protective IgG response, increased CD4+/CD8+ levels, or increased Th1-specific cytokines[144,145]. Cp15, a 15kDa surface protein, has been targeted as a potential antigen[146]. A recombinant vaccine targeting Cp15 has demonstrated protective responses in offspring of goats[145,147-150]. Additionally, Cp23, recognized as an immunodominant antigen, has several studies that show humoral and cellular response as well as a reduction in parasitemia in animal studies[151-154]. Other studies including mucin proteins and ribosomal proteins are currently being studied for potential vaccine development[147,152,154]. Currently, combination DNA vaccines include expression of *C. parvum* surface proteins such as Cp12 and Cp21 which have demonstrated an increase in T cell responses[144]. Another vaccine utilizes Cp15 and p23, with elevated Th1 cytokines observed in animal models[145].

However, there are also concerns regarding the efficacy of recombinant vaccines. For example, one recombinant vaccine, expressing Cp15 in a Salmonella vector, was no better than the vector alone[150]. Similarly, a recent study found that priming with *C. parvum* infection in was more effective in eliciting a protective immune response than vaccination strategies that use *Cryptosporidium* antigens and bacterial vector[155]. These studies, along with other similar observations, suggest that a live-attenuated vaccine may be more effective in providing protection against infection. Live-attenuated vaccines have long been shown to elicit a strong Th1 biased immune response and protective cellular immunity[156]. In *Plasmodium falciparum* for example, a live-attenuated vaccine is currently in advanced clinical trials[157]. Ideally, a vaccine for cryptosporidiosis would elicit a strong mucosal response[95,97,143]. Interestingly, a recent study found that priming with *C. parvum* in murine model of infection was more effective in eliciting a protective immune response than vaccination strategies that use *Cryptosporidium* antigens and bacterial vector[155]. Live-attenuated vaccines may be able to stimulate a robust immune response. One study attenuated *C. parvum* by γ-irradiation which reduced the viability and infectivity of parasites. Attenuated parasites were found to confer protection in calves[158].

## Research efforts in *Cryptosporidium*

### *Cryptosporidium* Research

Methods of cultivating *Cryptosporidium in vitro* are currently suboptimal. Woodnansee and Pohlenz[159] described the support of the entire life cycle *in vitro*[117,160], however the cell lines were only able to support infection for a few days, with a peak at 2-3 days post-infection and a decline was observed at the start of sporogony[159-162]. Even with high inoculation of oocysts, only a small number of parasites manage to develop into further stages[159]. This lack of development may be due to the import of essential nutrients by the parasite that may affect the life cycle

progression[163-165]. *Cryptosporidium* parasites lack the ability to synthesize several essential nutrients *de novo*, such as fatty acids, purines and amino acids[166]. Several efforts describe novel methodologies for the long-term cultivation and propagation of *Cryptosporidium*. One study utilizes a hollow-fiber technology which mimics the intestinal environment by delivering nutrients and oxygen from the basal layer upwards. This system reportedly maintained the production of viable and infectious oocysts for up to six months[167]. Similarly, a group recently described a 3-dimensional culture system derived from murine colonic explants. This system led to the development of neoplastic lesions *in vitro* when co-cultured with *C. parvum*, suggesting a role of parasites in carcinogenesis[168]. Traditionally, *Cryptosporidium* parasites have been studied using intestinal cells lines. One group in particular sought out to study seven different cells lines and found esophageal squamous-cell carcinoma cells (COLO-680N) produce viable oocysts that challenge the current standards for *in vitro* culture[169]. Another novel method utilizes stem-cell organoids, small 3-dimensional clusters of cells that mimic the intestine, to propagate *C. parvum* parasites[170]. These models resemble the gut in the development of microvilli, tight junctions, and the differentiation of an apical and basal end[171,172]. Without a method to continuously produce mature and infectious *Cryptosporidium* oocysts, researchers are not able to cultivate and store stocks of clones, which delay the progression of studies on host-pathogen interactions and late-stage genetics.

## Apicomplexan Egress

### Egress in Apicomplexans

*Cryptosporidium* is an obligate intracellular pathogen. After the initial stage of merogony, merozoites egress from host cells and infect neighboring epithelial cells to either complete the life cycle or propagate infection. In apicomplexans, egress is a fundamental step in the life cycle and

has recently been identified as a parasite-mediated process[173]. Protozoan egress tends to induce host cell lysis that contributes to disease pathology, including damage to tissues and subsequent inflammatory response[174]. The apical complex which harbor micronemes, specialized secretory organelles, that are mobilized for motility, invasion, and egress[174]. Mobilization of these organelles is promoted by increased cytosolic calcium levels[174-176]. However, the molecules responsible for this cascade have only recently been identified.

Others have proposed targeting molecules involved in egress as potential drug and vaccine candidates for apicomplexans[174,177-182]. For *Toxoplasma gondii*, a closely related apicomplexan, calcium-dependent protein kinase (CDPK3) is required for calcium-induced egress [183-185]. Interestingly, CDPK3 was not essential for gliding motility or invasion [174,183-185]. Furthermore, cGMP-dependent protein kinase G (PKG) is also an essential trigger for egress, and perhaps as a part of the same cascade as CDPK[174,186]. In this model, microneme secretion can be triggered by PKG, independent of calcium. PKG may be acting on the same substrate as TgCDPK3 and thus work together to facilitate egress[186].

The homologue of TgCDPK3 in *Plasmodium falciparum*, PfCDPK5, was also found to be crucial for egress but not required for invasion[187]. However, the cascade in *P. falciparum* differs from that in *T. gondii* in that PfCDPK5 appears to function downstream of PKG[188]. Another molecule identified in *P. falciparum* egress is subtilisin-like serine protease 1 (PfSUB1). It facilitates egress by acting on the parasitophorous vacuolar membrane[179,181]. PfSUB1 resides in parasite exonemes, which when secreted, cleave and activate putative proteases called SERA, which degrade the parasitophorous vacuolar membrane, host cytoskeleton, and host plasma membrane.

Egress is *Cryptosporidium*

Little is known about egress in *Cryptosporidium*. Due to the lack of methods to genetically modify the parasite, there has also been little advance in this area. A few studies have explored the expression patterns of key molecular players during *in vitro* infection. However, studies that delve into the specific role of these molecules have not been carried out. We propose to study the genes upregulated in the intracellular stages of infection using a novel silencing method developed in our lab. The aim of this project is to clarify the mechanism of egress in *C. parvum*. The key molecules involved in egress could provide targets for attenuation in the future development of a live-attenuated vaccine.

Our initial approach was to study the *C. parvum* orthologues of molecules that mediate egress for other apicomplexans. The recent methodological developments in genetic research for *C. parvum* have opened the doors to study many processes previously elusive. This project proposes to identify key molecules that have been identified in apicomplexan egress. **Hypothesis:** Calcium-dependent protein kinase 5 (CDPK5), subtilisin-like serine protease (SUB1), and cGMP-dependent protein kinase G (PKG) are essential for egress of *Cryptosporidium parvum* merozoites from host cells.

## CHAPTER 2: METHODOLOGY

## Gene Silencing in *Cryptosporidium*

Until recently, there have been no accepted methods for the genetic manipulation of *Cryptosporidium*. However, recent studies have described novel methods to advance the knowledge of *Cryptosporidium*. our group described adaptation of the RNA interference (RNAi) method to study *C. parvum* genetics[2]. Traditional RNAi methods utilize double-stranded RNA sequences (~19-21 nucleotides in length) transfected into target cells. RNA silencing will then rely on the cells' capability to recognize the double-stranded RNA fragment, bind, unwind the strands, and utilize the remaining guide strand to find target mRNA and cleave it to inhibit protein expression. Interestingly, *Cryptosporidium* lacks the complex which performs the RNAi processing[165]. Studies to define the processing complex (RISC) components, found that an RNase enzyme Argonaute 2 (Ago) played a fundamental role in the effector function of the complex[189]. Argonaute 2 loaded with the guide RNA can locate target mRNA and subsequently cleave the strand for degradation, in a complex called the minimal RISC[189].

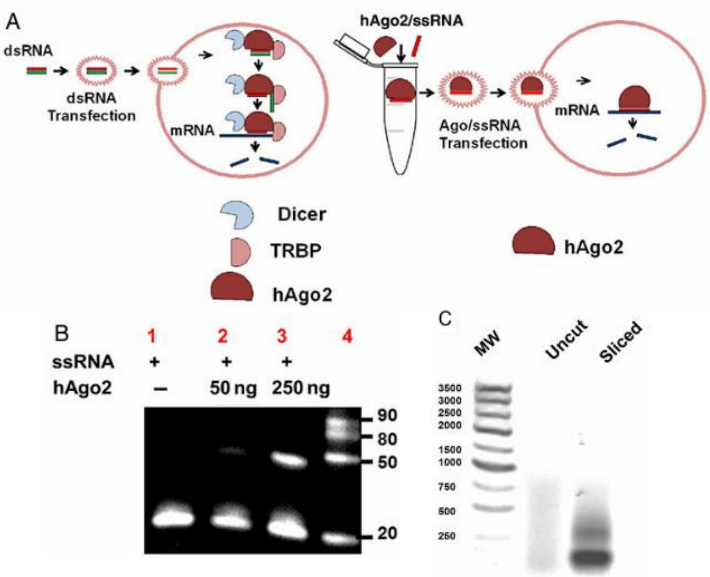


**Figure 3.** RNAi in *Cryptosporidium*. **A)** Schematic of silencing method for *Cryptosporidium*. On the left, a schematic representation shows RNAi in human cells, where intracellular components process miRNA for gene expression silencing. *Cryptosporidium,* however, lacks the processing components. This method utilizes human Argonaute 2, bound to single-stranded RNA, to target and silence gene expression. B) Assay used to determine complex formation. On lanes 1-3 samples are incubated with varying concentration of hAgo2. C) Electrophoresis to demonstrate parasite with complex (sliced) and without (uncut). Adapted from Castellanos-Gonzalez *et al.* 2016[2].

Based on these observations, our group assembled a minimal RISC with guide strands that were identified along target genes (Fig 3A). An electrophoretic mobility shift assay (EMSA) assay was used to determine the levels of protein-RNA binding and determined that 250 ng of Ago was ideal to produce a binding complex (Fig 3B)[2]. Slicing specificity was determined with RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE), an assay used to amplify RNA fragments (Fig 3C). Sliced mRNA is ligated to an adapter and subsequently amplified and sequenced[2]. The cleavage site was determined to be at the site where complimentary single-stranded RNA was designed. Transfection of the minimal RISC into *Cryptosporidium parvum* led to silencing of target genes (Fig 4A). Subsequent experiments also evaluated the levels of parasite invasion by silencing proteins that have been identified as key mediators of host cell entry. Experiments demonstrated a significant reduction of intracellular parasites when molecules involved in invasion (Cp15 and CDPK1) were silenced. By contrast, silencing of molecules involved in other stages (e.g. Gp900, Cp17, and Ino-5mon) showed no significant reduction (Fig 4B). This work provided a tool to identify molecules that are necessary for excystation, invasion, gliding motility, parasite development, and egress.

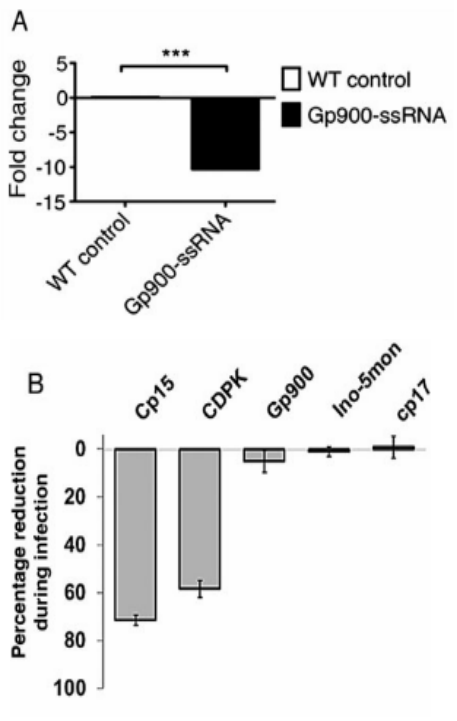


Figure 4. Efficacy of Adapted RNAi in *Cryptosporidium*. A) Silencing in mRNA. RT-PCR data shows a significant difference in silenced mRNA (Gp900) in comparison to wild-type controls. B) Silencing efficacy on infection. Several gene products were targeted for silencing. Cp15 and CDPK1 loss have the most impact on infection with ~70% and ~60% loss, respectively. Other targets were not observed to be effective for invasion using this model. Adapted from Castellanos-Gonzalez *et al*, 2016.

The use of an adapted RNAi method has also some implications for the field of drug development against cryptosporidiosis. Calcium-dependent protein kinases (CDPKs) are essential for intracellular calcium flux in apicomplexans[190]. Apicomplexan CDPKs have a distinct

structure from mammals, where the activation site lacks a gated region that typically protects the hydrophobic pocket of the enzyme[191,192]. This unique structure provides an attractive target for drug development[191,193]. Bumped-kinase inhibitors (BKIs) have been designed specifically to target CDPKs in *Cryptosporidium* without interacting with host cell enzymes[194]. Several studies have characterized the efficiency of these inhibitors in *C. parvum* as well as *T. gondii*[194-196]**.** One study in particular, looked at BKIs in both *in vitro* and *in vivo* models for *Cryptosporidium*[190].

*In vitro* testing of BKIs was carried out in *C. parvum* infected HCT-8 cells. A BKI of interest, 1517, was selected for further testing based on potency observed in previous experiments. The optimal concentration of anti-cryptosporidial activity of 1517 was determined by quantifying the reduction of parasites after exposure to the compound. Results indicated that a low dose (0.05 μM) was required to reduce the level of parasites in infected cells. For *in vivo* testing, *C. parvum* was used to infect SCID/beige mice, which were then treated with 1517 at differing concentrations and times. Oocyst shedding in stool was used to determine effectivity in comparison to vehicle controls. Mice were treated with 60 mg/kg once daily, 120 mg/kg once daily or altering 20/40 mg/kg twice a day. Results of these experiments showed that a regimen of once a day 120 mg/kg was most effective at reducing the number of parasite in stools. To demonstrate the specificity of BKIs 1517 and 1294 on CDPK, RNAi was used in conjunction with the BKIs. The experiment tested the hypothesis that if BKI was specific to CDPK, then an RNAi specific for the same target would have an additive silencing effect, which was indeed observed. This study not only demonstrated the feasibility of targeting CDPK1 for anti-cryptosporidial drug development, but also how RNAi can be a useful determinant in assuring the specificity of therapeutics, which can have off-target activity that may be harmful for the host[190].

Parasites, Infection Model, and Excystation

*Cryptosporidium parvum* oocysts, Iowa strain II, were purchased from the Animal and Comparative Biomedical Sciences department at the University of Arizona. Excystation of *C. parvum* was induced by incubating parasites with acidic water (60 µl, pH ~2.5) followed by 250 µl of 0.8% taurocholate in serum-free RPMI media as previously described[2]. Sporozoites were separated from unhatched and residual oocysts by filtration using a 3 µm-pore sized membrane (Millipore Sigma, Burlington, MA). *In vitro* infection was carried out with human colorectal adenocarcinoma epithelial cells (HCT-8; ATCC, Manassas, VA) as described previously[131,133,197]. Briefly: approximately $5x10^5$ HCT-8 cells were plated overnight on 24-well plates (Corning, Tewskbury, MA) to approximately 80% confluency, after which media was removed and *C. parvum* sporozoites (~$2x10^6$) in excystation media were added to establish a basal infection (2 hours at 37°C, 5%$CO_2$). For RNA extraction, cells and 250 µl of supernatant were collected at various times post-infection: 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 26 hours. For RNA extraction and silencing experiments infected cells were lysed directly from culture plates by adding 350 µl of Lysis buffer from Qiagen RNeasy Kit (Qiagen, Valencia, CA), while supernatants were passed through a 3 µm pore-sized filter to separate parasite from host cells and then supernatant was mixed with 350 µl of the Qiagen lysis buffer, and stored at -20°C.

Antisense Single Strand RNA (ssRNA) Design

Single-stranded antisense RNA sequences, 19-29 nucleotides in length, targeting different target regions of CpSUB1, CpCDPK5, and CpPKG were designed using RNAfold software (Institute for Theoretical Chemistry, University of Vienna). All antisense RNA contained modifications as previously described[2]. RNA was designed to target areas with hairpin and loop secondary structure low G/C count, lack of internal repeats, and within a 500 b.p. distance from start codon. Single-stranded antisense sequences are listed in Table 1. Target sequences were: CDPK5 (cgd2_1300; XP_626355.1), SUB1 (cgd6_4840; XP_627811.1), and PKG (cgd8_750; XP_626985.1).

447 Silencing of RNA Expression in Oocysts

448 To carry out silencing experiments, 250 ng of purified recombinant human Argonaute 2
449 (hAGO2) (Sino-Biological, Beijing China) was coupled with single-stranded RNA (1 µM) with
450 assembly buffer (30 mM HEPES, pH 7.4, 150 mM potassium acetate, and 2 mM $MgCl_2$)[2,198] and
451 incubated at RT for 1 hour. To encapsulate the complex, 10 µl of Protein Transfection Reagent
452 (PTR) (Thermo Fisher Scientific, Waltham, MA) were added to the sample and incubated at RT
453 for 30 minutes. For transfection experiments $5x10^5$ *Cryptosporidium* oocysts were added to the
454 sample containing the complexes and incubated at RT for 2 hours. To confirm silencing, ~20 ng
455 of total RNA was extracted from transfected oocysts using the Qiagen RNeasy kit (Qiagen,
456 Valencia, CA) and stored at -20°C until further analysis with real-time PCR.

457 Quantification of Egress by Flow Cytometry

458 To quantify the egress of merozoites from infected cells, we analyzed the supernatant by
459 flow cytometry. For these experiments, *C. parvum* oocysts were treated with Ago2-complexes as
460 described. After excystation, the sporozoites were incubated with cell tracker 0.5 µM
461 carboxyfluorescein succinimidyl ester (CFSE) (Thermo Fischer Scientific, Waltham, MA). HCT-8
462 cells were infected with $5x10^5$ sporozoites for 2 hours at 37°C to establish a basal infection and
463 then media was removed, and the infected cell layer was washed with ~250 µl PBS once. The
464 cells were then resuspended in RPMI media supplemented with 10% FBS and incubated for 26
465 hours. To evaluate the effect of silencing on egress, we quantified the number of labeled parasites
466 in the supernatant from infected cells. For these experiments, 300 µl of supernatant was collected
467 every 2 hours from 1-26 hours post-infection. We used flow cytometry to count stained viable
468 merozoites. We separated parasites from sloughed HCT-8 cells with a 3 µM filter. Samples were
469 then centrifuged at 8,000xg for 3 minutes and resuspended with 10 µl of 4% paraformaldehyde
470 in water and then in 500 µl of PBS. Samples containing stained parasites were analyzed by flow

cytometry, using the Stratedigm SE500 Analyzer (Stratedigm; San Jose, CA). Viable (CFSE-stained) merozoites were quantified using side-scatter and forward-scatter size exclusion to identify positively stained parasites.

Quantification of Merozoites (rtPCR)

Total number of parasites released in the supernatant was determined by rtPCR. The controls in these experiments were wild-type (untreated parasite) and scramble (nonsense ssRNA transfected parasites). We collected supernatant and extracted RNA at 1, 22, 24, and 26 hours post-infection (see parasites, infection model, and infection assay). To evaluated silencing efficiency, we compared gene expression of parasites with SUB1 silenced (ΔSUB1)/CDPK5 silenced (ΔCDPK5)/PKG silenced (ΔPKG), wild-type, and scrambled parasite. 100 ng of total RNA was used as a template with SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Life Technologies; Grand Island, NY), The following conditions were used: 55°C for 20 minutes, 95°C for 5 minutes, and 40 cycles at 95°C for 15 seconds and 65°C for 1 minute (AB7500 Fast RT-PCR System for 96-well plates). Primers are shown in Table 2. RNA expression of ΔSUB1, scrambled-ssRNA, and wild-type parasites were evaluated using the comparative Ct method to assess the relative expression of SUB1, CDPK5, or PKG to parasite lactate dehydrogenase (LDH, cgd7_480; XP_628238.1. All samples were analyzed in triplicate in at least two independent experiments.

Observation of Intracellular Parasites by Confocal Microscopy

To evaluate the effect of silencing ΔSUB1 on blockage of egress we evaluated the number of intracellular organisms at 24 hours post-infection by confocal microscopy. For these experiments HCT8-cells were grown on 22x22 mm coverslips until confluent. Approximately $5x10^5$ *C. parvum* oocysts, after silencing (ΔSUB1, ΔCDPK5, or ΔPKG) or wild-type, were used to infect host cells for 24 hours at 37°C 5%CO2. At the end of the infection period, supernatant was

removed, and infected cells were washed with PBS, fixed with 4% paraformaldehyde (room temperature for 25 minutes) then washed with PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, washed with PBS 3 times and stained with DAPI (VECTASHIELD Antifade Mounting Medium with DAPI; Vectashield, Burlingame, CA). Samples were then visualized using the Zeiss LSM 880 with Airyscan confocal microscope (Zeiss; Oberkochen, Germany).

## CHAPTER 3: CALCIUM-DEPENDENT PROTEIN KINASE 5 (CDPK5)

### CDPKs: Unique Enzymes in Apicomplexans

Published genomes for *Cryptosporidium*, *Plasmodium* and *Toxoplasma* have facilitated comparative studies to better understand host-pathogen relationships[199-201]. $Ca^{2+}$ stimulated molecules are conserved among apicomplexans[202,203]. Studies suggest that calcium is an important second messenger that regulates vital processes such as motility[204], protein secretion[176,205,206], and differentiation[207,208]. Interestingly, calcium-dependent enzymes in apicomplexans resemble plant enzymes[209]. For example, calcium-dependent protein kinases in animals general contain a $Ca^{2+}$Calmodulin (CaM)-dependent kinase site of activation[210]. These bulky groups serve as gatekeepers for the active site of the enzyme. Plants, on the other hand, have CDPKs that lack a calmodulin activation site, but still retain an analogous function[1,9]. Apicomplexans have a glycine instead of the gatekeeper residue, facilitating binding of inhibitors that are blocked by the gatekeeper residues. The unique structure of CDPKs in apicomplexans makes it a potential target for therapeutics against the pathogens in this phylum.

### CDPKs in Egress of Apicomplexans

CDPKs are adapted to control diverse intracellular responses [211]. Different member of the phylum has distinctive numbers of CDPKs: *T. gondii* 11, *P. falciparum* 8, and *C. parvum* 7[209]. CDPK1 in *T. gondii* (TgCDPK1) was first described as playing a fundamental role in gliding motility, host cell attachment and microneme secretion[212]. It was also suggested that it played a key role in egress, but it was later discovered that the role was non-specific[175]. In 2010, a group of researchers developed inhibitors to TgCDPK1 that also function to inhibit CDPK1 in *C. parvum* (CpCDPK1)[196]. These inhibitors were developed specifically for the glycine residue present at the activation site of apicomplexan CDPKs[213]. The results of this study marked an important role of these enzymes for invasion in their respective host cells[196].

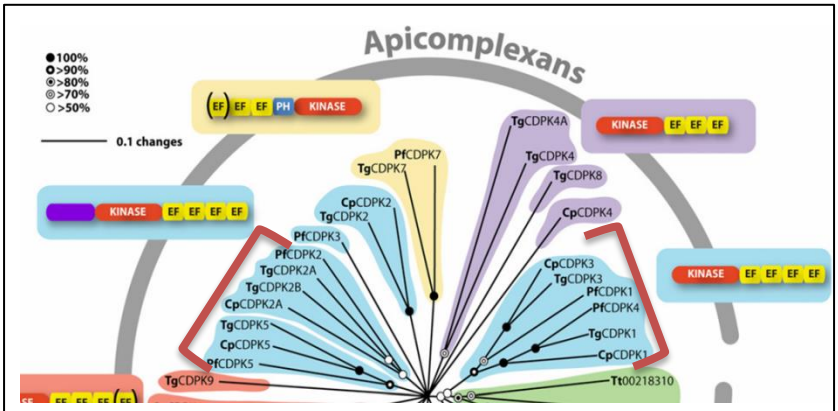


**Figure 5.** Phylogenetic relationship of CDPKs in apicomplexan parasites. The phylogenetic relationship of CDPKs in *Cryptosporidium parvum*, Toxoplasma gondii, and Plasmodium falciparum are represented. The CDPKs in red brackets highlight the CDPKs implicated in egress and the CDPKs that show close homology to them. Adapted from Billker *et al,* 2009.

TgCDPK3 plays a specific role in egress from host cells[184]. Similarly, studies indicated that PfCDPK5 regulates egress in blood stage malaria[187]. A comparative analysis studied the phylogenetic relationship among CDPKs in *Toxoplasma, Plasmodium* and *Cryptosporidium*[211]. This study indicated that CDPKs which played a primary role in egress in both *T. gondii* and *P. falciparum* belonged to a group of CDPKS that contained a structure consisting of four EF-hand motifs (Fig 5). Based on the close relationship to these CDPKs, we could predict which target may have a direct role in egress of *Cryptosporidium parvum*.

*Cryptosporidium* CDPK1 (CpCDPK1) is essential for invasion of host cells[196]. However, the CDPK responsible for egress of the parasite has yet to be described. There is a strong homology of TgCDPK3 with CpCDPK3 and PfCDPK5 with CpCDPK5[211,214]. Both TgCDPK3 and PfCDPK5 have been identified as moderators of egress. However, using NCBI Blast®, we found a higher sequence homology exists between PfCDPK5 and CpCDPK5 (35%) than between TgCDPK3 and CpCDPK3 (14%). Based on these observations, we predict that calcium-dependent protein kinases play a pivotal role in egress of *Cryptosporidium parvum*. **Hypothesis**: Calcium-dependent protein kinase 5 (CDPK5) of *C. parvum* is essential for egress of merozoites from host cells.

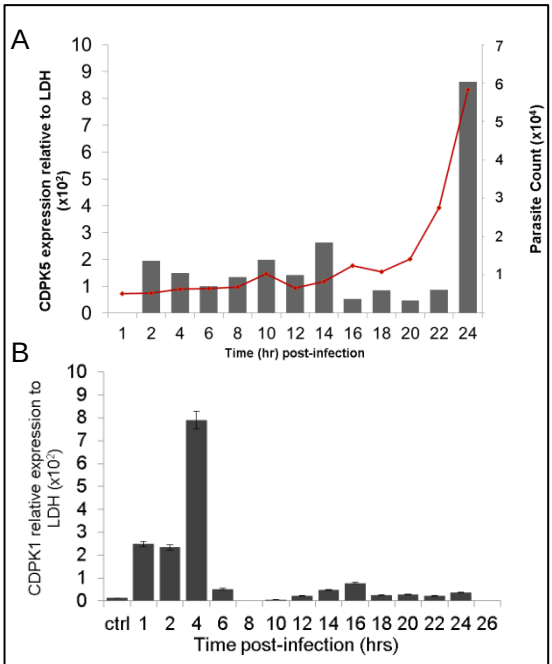


**Figure 6.** Egress and CDPK5 expression profile. A) Egress of merozoites from *in vitro* infection was determined from supernatants. At 24 hours post-infection a peak of egress is observed (red line). CDPK5 expression throughout infection was determined with RT-PCR. CDPK5 expression peaks at 24 hours post-infection at the timepoint of infection. B) Expression profile of CDPK1. In contrast to the expression profile of CDPK5, there is a distinct expression profile in CDPK1 where peak of infection is observed at 4 hours post-infection.

## Results

We began our study by first determining the time of merozoite egress. We infected confluent HCT-8 cells with sporozoites and incubated for 26 hours. We collected supernatant every two hours as well as the first hour post-infection as a control. Supernatants were filtered to remove any host cell debris, and parasite counts were determined by rtPCR compared to a standard curve. In this experiment, a gradual increase of parasites in supernatant was observed at 16 hours and peaked at 24 hours post-infection (Fig 6A).

We designed primers specific for CDPK5 to observe the levels of expression during merogony. As a control, we also used primers specific for CDPK1. We infected confluent HCT-8 cells with viable sporozoites and collected infected monolayers and supernatants at 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26 hours post-infection. Total RNA was extracted and rtPCR was used to amplify either CDPK5 or CDPK1 and expression patterns were determined relative to the housekeeping gene, LDH. There was low level expression of CDPK5 from 2-14 hours post-infection. After that, expression dropped until a major peak of expression was observed at 24 hours, coinciding with the timepoint of egress for this experiment (Fig 6B). In contrast, the expression pattern of CDPK1 had an increase from the beginning of infection, followed by a peak at 4 hours post-infection.

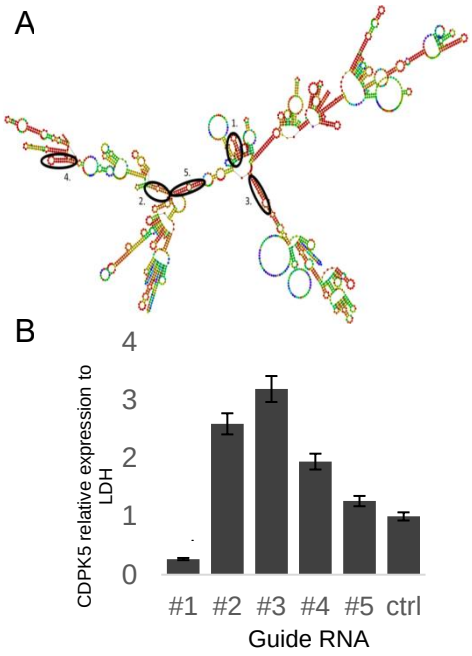


**Figure 7**. Silencing of CDPK5.A) Model of CDPK5 mRNA with target areas for siRNA silencing marked. The coloration depicts areas with high probability of siRNA binding (red) to low probability (blue). B) Silencing of CDPK5 in *C. parvum* oocysts. Oocysts were silenced with CDPK5-specific ssRNA and hAgo2. Total RNA was extracted and expression of CDPK5 was determined with RT-PCR amplification relative to LDH.

We designed five single-stranded RNA segments complimentary to CDPK5 (Fig 7A). We incubated oocysts with the silencing reaction and ssRNA. After incubation, we extract total RNA and amplified CDPK5 and compared expression to the housekeeping gene, LDH. From these experiments, we observed potent silencing with guide RNA #1 (Fig 7B). The other guide RNAs had no significant effect on CDPK5 expression.

To measure the effect of CDPK5 silencing on egress, we silenced CDPK5 expression in oocysts. We then infected confluent HCT-8 cells and incubated for a total period of 26 hours. We collected supernatant at 22, 24, and 26 hours post-infection, to coincide with potential timepoints of egress. We also collected supernatant at 1 hour post-infection as a control. We extracted total RNA and amplified CDPK5 relative to LDH as mentioned before. The results showed a peak of parasite release at 24 hours post-infection in wild-type and ΔCDPK5 samples, which coincided with the time of egress. There was no significant difference the in the quantity of parasites collected at any timepoints (Fig 8A). Additionally, we used flow cytometry to quantify parasites released in the supernatant after 24 hours post-infection. We observed similar values for CFSE+-stained parasites in wild-type controls versus Δ-CDPK5 silenced parasites (Fig 8B).

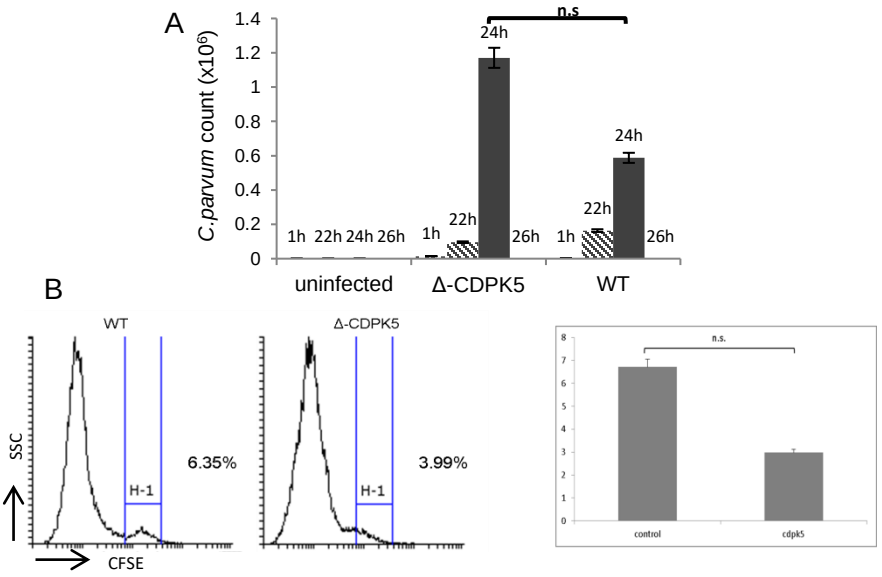


Figure 8. Δ-CDPK5 parasites in egress. A) Using RT-PCR, we determined the levels of parasites collected in supernatant at the timepoint of egress at 1hr, 22hr, 24hr, and 26hr post-infection. There was no significant difference between wild-type and silenced parasites. B) Using flow cytometry, we quantified CFSE+ stained parasites in supernatant at 24 hours post-infection and found no significant difference between samples.

## Conclusion

We have observed the time of egress *in vitro*. We also observed the expression patterns of CDPK5 which included a drastic peak at the time point of egress. Based on these results, we designed a unique ssRNA that demonstrated potent silencing in viable oocysts. However, we observed no significant decrease in merozoites released with Δ-CDPK5 parasites compared to controls. Overall, this experimentation indicates that CDPK5 is not necessary for egress in *Cryptosporidium parvum*.

## CHAPTER 4: SUBTILISIN-LIKE SERINE PROTEASE 1 (SUB1)

SUB1 in Apicomplexans

Subtilisin-like serine proteases (subtilases) are responsible for a number of post-translational proteolytic processing of proteins in invasion and egress in apicomplexans[174]. Apicomplexan subtilases are more structurally similar to subtilisin in *Bacillus subtilis*, a Gram(+) bacterium, than serine proteases in higher eukaryotes, which have drawn attention as a potential therapeutic[215]. Moreover, the catalytic domain is highly conserved among apicomplexans[216]. Apicomplexans all contain specialized secretory organelles, which are often utilized for key processes in the parasite lifecycle such as invasion, motility, and egress. Evidence demonstrates that serine proteases are discharged from exonemes, apical organelles, into the PV lumen prior to egress[73,189,216].

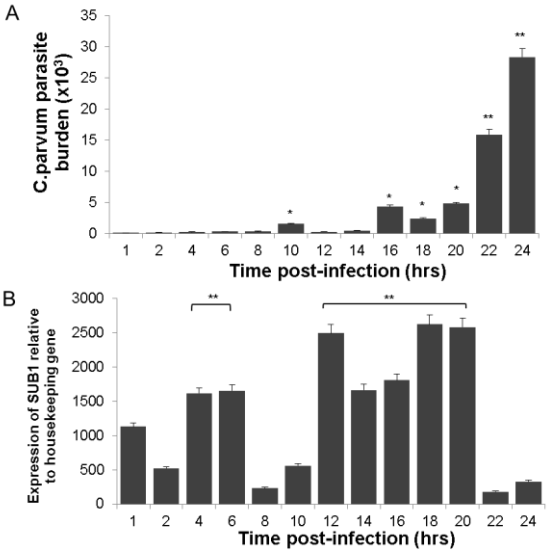


Figure 9. Timing of parasite egress and SUB1 expression. Viable *Cryptosporidium parvum* sporozoites were used to infect HCT-8 monolayers. A) Supernatants were collected and assayed for released parasites using qRT-PCR. The peak of released merozoites was observed at 24 hours post-infection (**p<0.0001 and *p<.005). B) Sporozoites infected HCT-8 monolayers were collected at different times post-inoculation. Peak of expression for SUB1 was observed at 18-20 hours post-infection with respect to initial infection at 1 hour. (Nava, *et al.* in preparation).

Subtilisin-like serine protease (SUB1) have been recently identified as key modulators of egress in *Plasmodium*[174]. This endoprotease is discharged in the parasitophorous vacuole (PV) lumen prior to egress[174]. SUB1 proteolytically cleaves substrates including merozoite surface proteins and papain-like proteases called serine repeat antigens (SERA) [217-219]. Cleavage of merozoite surface proteins leads to activation of invasion proteins. SERA proteins are cleaved to release their papain-like domains, which degrades the PV membrane as well as host cytoskeleton[220,221]. *Plasmodium* has three distinct

subtilisin-like serine proteases (SUB1, SUB2, and SUB3), SUB1 is the only one implicated in egress of merozoites both in the erythrocytic and liver stages of the life cycle[181]. *Toxoplasma gondii*, SUB1 is found to activate microneme-adhesive proteins, which link SUB1 activity to microneme secretion[222]. A recent study demonstrated that genetic mutation of TgSUB1 does not affect egress[223].

SUB1 in *Cryptosporidium*

In contrast to *P. falciparum* and *T. gondii*, there is only one SUB1 gene found within the genome of *C. parvum*[224]. SUB1 was initially identified as a key protease in invasion of *Cryptosporidium* in host cells. SUB1 cleaves gp40/15, a precursor glycoprotein that facilitates invasion[225,226]. While SUB2 in *Plasmodium* is crucial for invasion, the lack of multiple subtilisin-like proteases in *Cryptosporidium* could suggest multiple roles for this single enzyme. Expression patterns of CpSUB1 in *in vitro* infection were upregulated at timepoints coinciding with the development and release of merozoites[225,227]. However, studies in *Cryptosporidium* have not studied the role of CpSUB1 during egress. Based on these collective observations, we **hypothesize** CpSUB1 is essential for egress of *Cryptosporidium parvum* merozoites.

Results

**SUB1 expression peaks prior parasitic release from in vitro culture.** To determine the timepoint of parasite egress from human epithelial cells, supernatants from infected cells were collected every 2 hours for the first 26 hours post-infection. Merozoite release was determined by measuring parasite mRNA in supernatants. Parasite RNA appeared at 18 hours with highest levels detected at ~24 hours post-inoculation (Fig 9A). We found that SUB1 mRNA expression

peaked at 18-20 hours post-infection in samples of infected cells and supernatant, with a significant drop occurring at 22-24 hours (Fig 9B). As a control, we determined the expression of another serine protease from the subtilase family (cgd2_3660; XP_001388237.1) and observed a peak at 1 hour post-infection with no significant increase in expression at subsequent timepoints.

**Silencing of SUB1 mRNA Expression.** Using base-pairing probability algorithms determined by predicted secondary structure, we synthesized five guide sequences designed to optimize binding to SUB1 mRNA (Fig 10C). Out of the five guide sequences targeting *Cryptosporidium* SUB1, antisense sequence #4 and #5 resulted in an 80% ($p<0.0001$) and 60% (and $p<.05$) reduction in SUB1 mRNA compared to wild-type, respectively. By contrast, guide RNA #2 was not effective, and guide RNAs #1 and #3 were toxic to the parasites (Fig 10B). To confirm that silencing was not detrimental to parasite viability, we stained oocysts with CFSE after silencing and induced excystation. Using fluorescent microscopy, we observed that similar levels of excystation and staining with the vital dye CFSE with antisense RNA #4 compared to wild-type samples (Fig 10C). Thus, using guide RNA #4, we were able to silence expression of SUB1 without affecting parasite viability or excystation. Subsequent studies used this guide RNA to create parasites with silenced SUB1 (ΔSUB1).

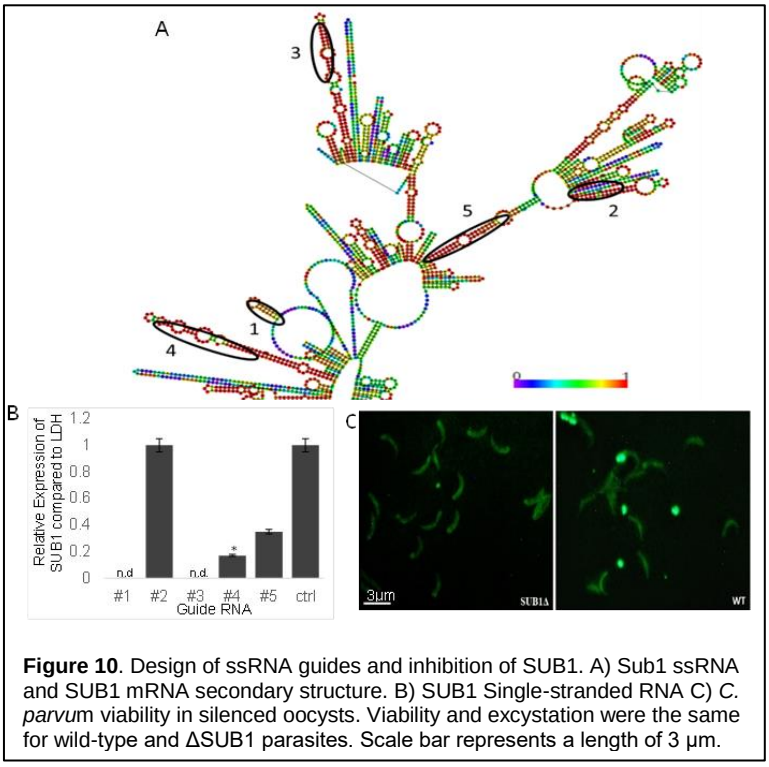


**Figure 10**. Design of ssRNA guides and inhibition of SUB1. A) Sub1 ssRNA and SUB1 mRNA secondary structure. B) SUB1 Single-stranded RNA C) *C. parvu*m viability in silenced oocysts. Viability and excystation were the same for wild-type and ΔSUB1 parasites. Scale bar represents a length of 3 μm.

**SUB1 blocks parasite egress from host cells.** With ΔSUB1-sporozoite infection, there was a 95% decrease ($p<0.0001$) in parasite RNA in supernatants of infected HCT-8 cells compared to wild-type parasites (Fig 11A). Decreased egress was also confirmed by flow cytometry (Fig 11B). Three independent experiments demonstrated a 99.5% decrease of ΔSUB1 samples in comparison to wild-type controls ($p<0.0001$).

We used confocal microscopy to visualize retained intracellular meronts in host cells. The infected cells were stained with DAPI and visualized using confocal microscopy. ΔSUB1-*C. parvum* infected cells demonstrated increased levels of intracellular parasites compared to wild-type controls (Fig 11C). To quantify the parasite burden in host cells after the time of egress we used rtPCR of infected cells. At 24 hours post-infection, there is a higher level of parasites in ΔSUB1-silenced samples compared to wild-type controls (Fig 11D).

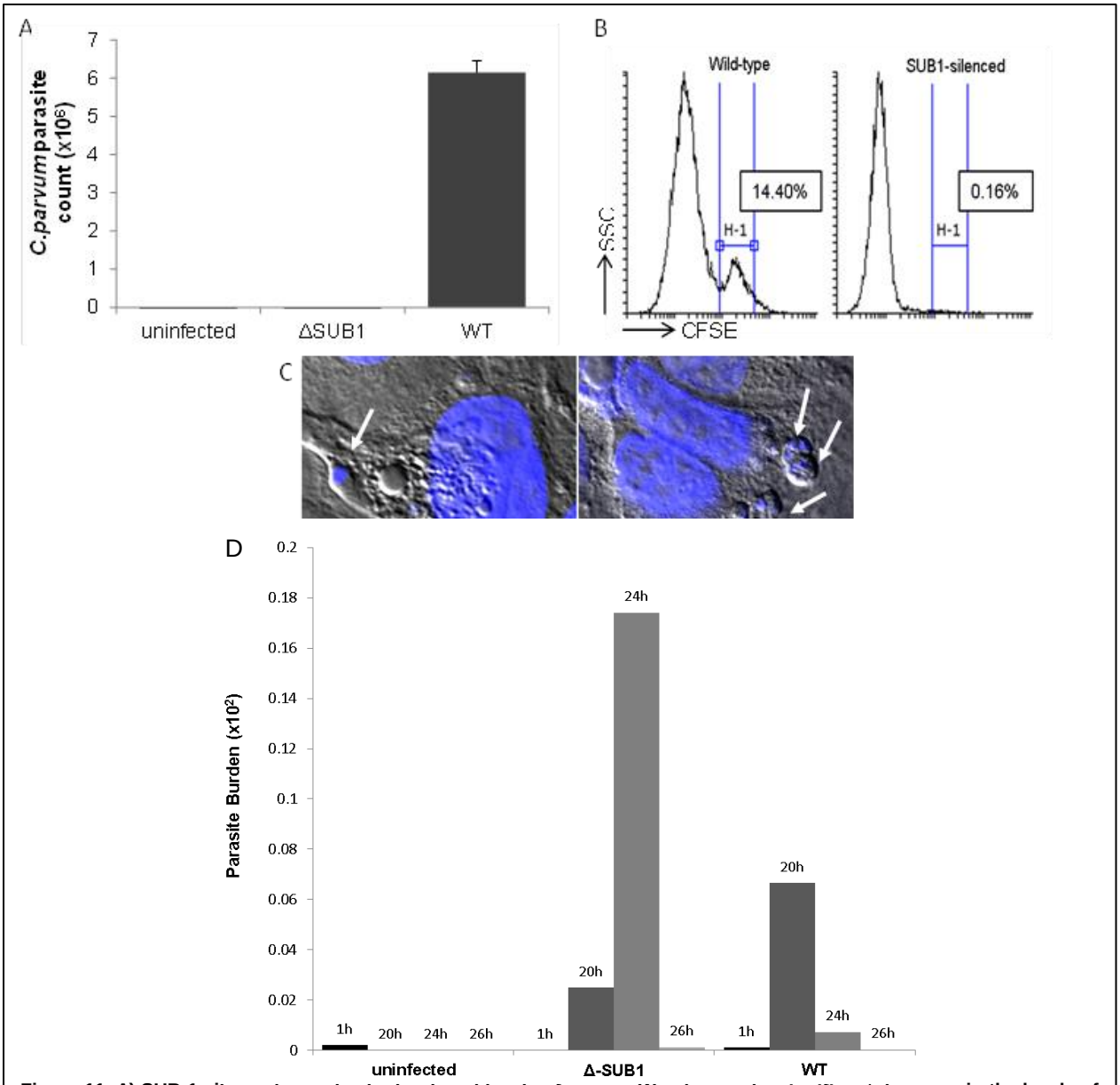


**Figure 11. A) SUB-1 silenced samples had reduced levels of egress. We observed a significant decrease in the levels of egressed parasites from collected supernatants compared to controls at 24 hours post-infection. B) Quantification of free merozoites after silencing. Supernatant of infected cells was collected, and parasites were stained prior to infection. Samples were analyzed and quantified with FACS. The proportion of stained free merozoites is highlighted in H-1 subpopulation and found to be significantly decreased in ΔSUB1- parasites compared to wild-type controls. C) *C. parvum* infected HCT-8 cells stained with DAPI and visualized using confocal microscopy. ΔSUB1 parasites demonstrated an increased number of intracellular parasites compared to wild-type controls. D) Intracellular parasite burden. Burden of intracellular parasites was determined with rtPCR amplification of a parasite housekeeping gene. At 24 hours post-infection there is an increased level of parasites in ΔSUB1 parasites compared to wild-type controls.**

690

691 Conclusions

To evaluate the role of SUB1 during the egress, we found that mRNA expression of CpSUB1 peaked at 18-20 hrs. post-infection. Silencing of CpSUB1 expression significantly decreased merozoite egress when compared ΔSUB1 to wild-type controls. Additionally, we also confirmed these observations using flow cytometry. We used confocal microscopy and qPCR to confirm that parasites were retained parasites within host cells.

## CHAPTER 5: cGMP-DEPENDENT PROTEIN KINASE G (PKG)

Role of PKG

PKGs (cyclic GMP-dependent protein kinase G) are kinases that target serine-threonin specific proteins and have a structurally distinct activation site. This unique structrues has drawn considerable attention as a therapetic target[179,227]. The unique structure of PKG is conserved among apicomplexans, and consists of a small gatekeeper residue as well as two allosteric activation sites. In contrast to mammalian and *Drosophila* PKGs, which have a large gatekeeper reside and three allosteric activations sites[227-229].

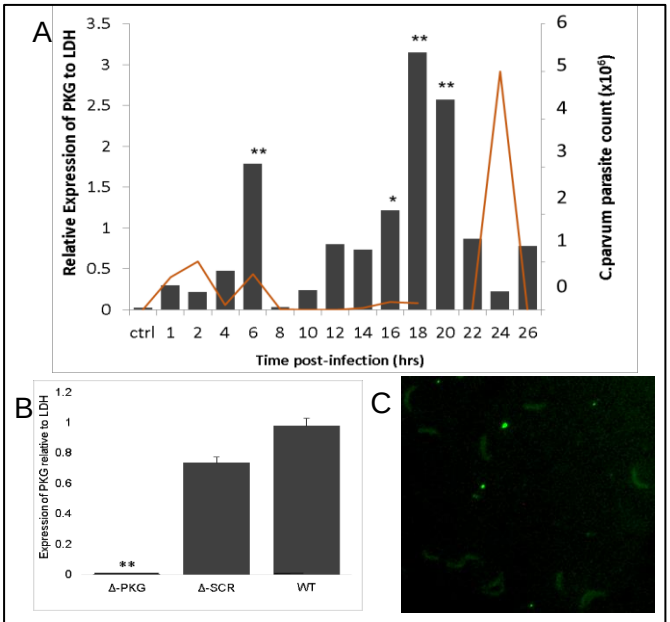


**Figure 12**. PKG expression and silencing. Expression pattern of PKG post-infection and time of egress of Cryptosporidium merozoites into the supernatants (red line). An increase in PKG was noted at 6 hours post-infection and peak expression at 18-20 hours post-infection (***p<0.0001, *p<0.01). B) Silencing of parasite Protein-kinase G. The ratio of parasite PKG to LDH mRNA was markedly reduced by transfection with a PKG-specific ssRNA bound to Argonaute compared to scrambled-antisense and wild-type controls (~99%) (***p<0.0001). C) Viability of Δ-PKG was confirmed by staining with the vital dye carboxyfluorescein succinimidyl ester (CSFE) and by excystation.

PKG plays a central role in egress of other apicomplexans[179]. In *Plasmodium*, PKG is considered a central regulator of microneme secretion[179,230]. PKG plays a significant role in egress for both erythrocytic as well as liver stages, where inhibition of activity leads to a block in $Ca^{2+}$ mobilization and decreased egress and invasion[230-232]. The mobilization of $Ca^{2+}$ from intracellular stores led to cGMP-dependent protein kinase G (PKG) mediated exonemes/microneme secretion[179,220,222,233]. In *Toxoplasma gondii*, PKG mediates egress, motility and invasion of host cells, all of which rely on microneme secretion[227,234]. PKG deletion is fatal to the parasite[227]. PKG can partially compensate for the effect of inhibition of CDKP3 on egress[183]

PKG in *Cryptosporidium*

We identified the orthology of PKG of *T. gondii* and *P. falciparum* in *Cryptosporidium*[2352352352342332332322262 25]. **Hypothesis**: cGMAP-dependent protein kinase G is essential for egress of *Cryptosporidium* merozoites from host cells. In this study, we test the hypothesis that PKG plays a key role in egress of *Cryptosporidium* merozoites from host cells.

Results

**PKG mRNA peaked shortly prior to merozoites egress.** When *Cryptosporidium* sporozoites infected HCT-8 cells, there was a transient increase of PKG expression at 6 hours post-infection (Fig 12A). PKG mRNA then peaked at 18-20 hours post-infection. This peak expression of PKG mRNA preceded egress by a few hours as would be expected for molecules leading to egress.

**Silencing of PKG in *Cryptosporidium parvum* does not affect viability of parasite excystation.** To interrupt PKG expression in *Cryptosporidium*, single-stranded antisense

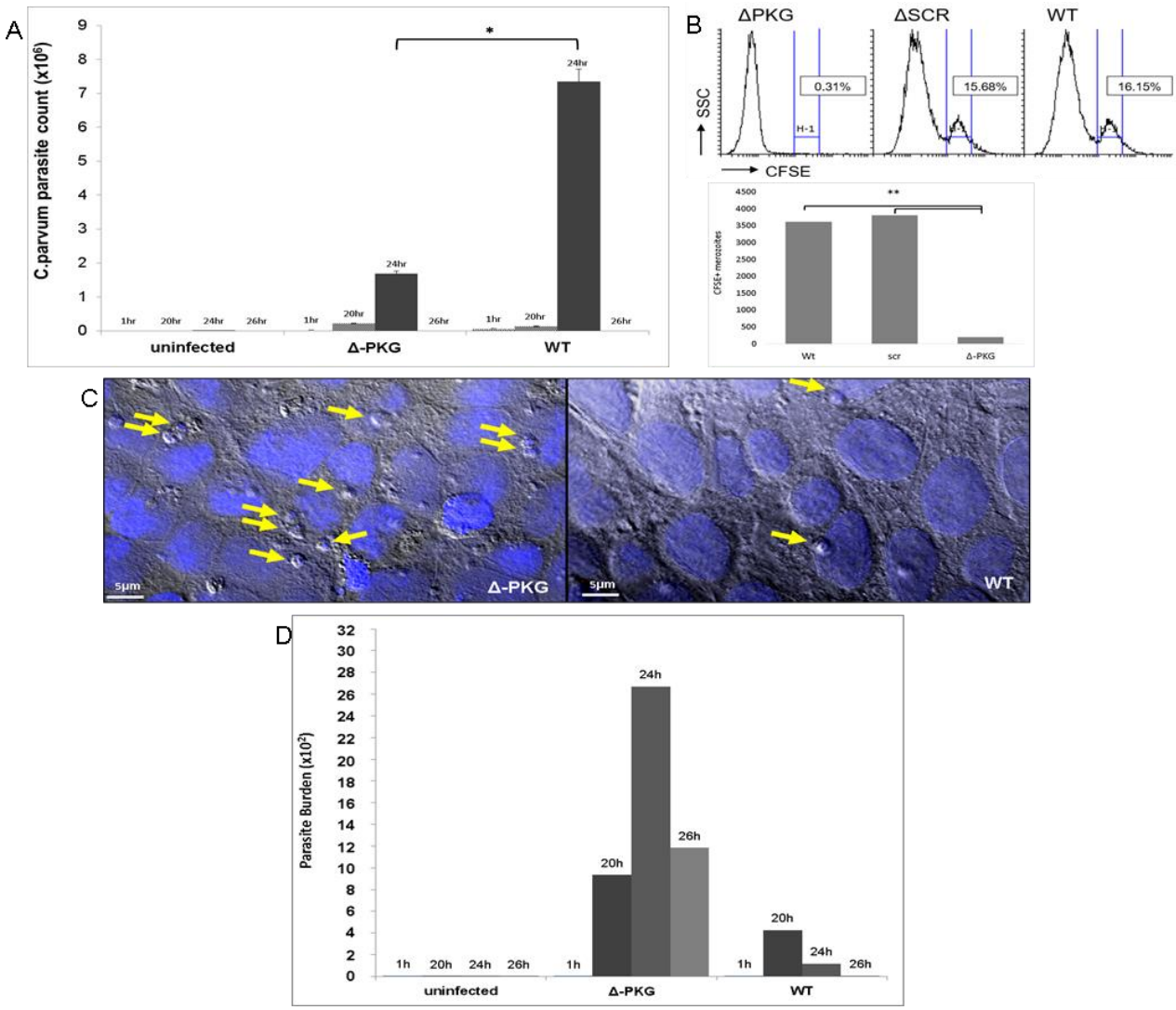


**Figure 14**. Merozoite egress in Δ-PKG parasites. A) Parasite count demonstrated in supernatant of timepoints surrounding the timepoint of egress. The peak of egress at 24 hours post-infection was significantly ($p<0.05$) decreased in Δ-PKG compared to in wild-type parasites. Furthermore, egress is neither delayed nor advanced in silenced parasites as noted at 20 and 26 hours post-infection, which display similar numbers to wild-type samples. B) Sporozoites were stained with CFSE prior to infection. At 24hours post-infection, merozoites in the supernatant were quantified using flow cytometry. Scrambled-ssRNA and wild-type controls showed similar levels of parasites (15.68% and 16.15%, respectively). However, Δ-PKG parasites were significantly reduced (0.31%). C) Infected HCT-8 cells were stained with DAPI and visualized with confocal microscopy. ΔPKG-*C. parvum* parasites demonstrated higher levels of trapped parasites within host cells compared to wild-type or scrambled controls. D) *C. parvum* merozoites were quantified in host cells using rtPCR quantification of a housekeeping gene. At 24 hours post-infection, higher levels of parasites were demonstrated in Δ-PKG parasites compared to WT controls, which was observed until 26 hours post-infection. (Nava *et al,* submitted).

sequences were designed. We tested potency of guide sequences to silence PKG expression in viable oocysts by measuring the ratio of PKG relative to the parasite-housekeeping gene (LDH) by qPCR. Δ-PKG parasites exhibited a 98-99% reduction in PKG:LDH mRNA ratio compared to

wild-type (Fig 12B). To confirm that loss of PKG did not affect parasite viability, we induced excystation and determined viability with the vital dye CFSE. In comparison to wild-type and scrambled-ssRNA controls, Δ-PKG oocysts did not have a reduction in excystation or CFSE staining (Fig. 12C). Thus, decrease of PKG expression in *C. parvum* oocysts, did not affect viability or excystation of the parasites.

**Δ-PKG merozoites do not egress from host cells.** To determine the effect of PKG on egress, we infected HCT-8 cells with Δ-PKG or wild-type parasites. At 24 hours post-infection, Δ-PKG parasites express a significant decrease ($p<0.05$) in merozoites mRNA released into the supernatant (Fig. 13A). At 1 hour and 20 hours post-infection, there are minimal levels of parasites in supernatant in both Δ-PKG and wild-type samples. However, the wild-type parasites exhibit a peak of merozoite release at about 24 hours post-infection. By contrast, the Δ-PKG parasites did not exhibit a peak at any of the time points. To further confirm our results, we stained Δ-PKG parasites with CFSE prior to infection and quantified parasites released from host cells by flow cytometry. Wild-type and scrambled-ssRNA parasites had similar percentages of merozoites within the total population collected (15.7% and 16.2%, respectively). However, for Δ-PKG samples only 0.35% of cells were CFSE positive and within the merozoite gate (Fig 13B). Using confocal microscopy with DAPI staining, we demonstrated an increased number of intracellular parasites compared to wild-type and scrambled-ssRNA controls (Fig 13C). We used rtPCR of a housekeeing gene to determine the parasite burden that remained within host cells before and after the time of egress (Fig 14D). At 24 hours post-infection, the time of egress, there is a greater burden of parasites in Δ-PKG silenced parasties versus wild-type (WT) controls, which is maintained through 26 hours post-infection.

## Conclusion

We designed antisense guide RNA sequences to inhibit the expression of PKG in viable *Cryptosporidium* oocysts, and we were able to decrease the expression levels of PKG without

affecting parasite viability, excystation, or invasion. We then demonstrated that silencing of PKG significantly affected the ability of merozoites to egress from host cells. Additionally, we collected supernatants before and after the typical timing of egress and confirmed that egress was blocked, rather than advanced or delayed. Furthermore, we confirmed these results via flow cytometry of supernatants and confocal microscopy of the residual cell layer.

## CHAPTER 6: DISCUSSION

Egress is a key step in the *Cryptosporidium* life cycle. Parasites develop in a vacuole that is intracellular but also separate from the host cell cytoplasm. Thus, unlike many other intracellular pathogens, there is no direct spread from cell to cell. Instead, egress is essential for proliferation and development of disease. While *Cryptosporidium* invasion has been studied extensively [236-241], there is little information regarding the molecular mechanisms behind egress. We have identified *Cryptosporidium* homologues of some of the mediators of egress for related parasites. We predicted that these targets would have simlar function in facilitating escape of parasite from host cells. The work from this study demonstrates that PKG and SUB1 are crucial for *Cryptosporidium* merozoite egress (Nava et al, submitted for publication), as has previously reported for *Plasmodium* and *Toxoplasma*. In contrast, CDPK5 in *Cryptosporidium* did not seem to play a role in egress.

### Calcium-dependent Protein Kinases in Egress

Calcium-dependent protein kinases (CDPKs) are critical mediators of egress of related apicomplexans[209,211]. Intracellular calcium stores have been found to facilitate escape from host cells[175,211]. *Plasmodium falciparum* CDPK5 (PfCDPK5) facilitates egress of merozoites from both liver cells and infected red blood cells. CDPK5 in *C. parvum* (CpCDPK5) is nearly identical to

PfCDPK5, which led to our hypothesis that it may play a crucial role in egress of *Cryptosporidium* merozoites. In this study, CpCDPK5 had a peak expression at 24 hours post-infection, at the time of maximum egress. We optimized silencing of CDPK5 mRNA, however, the quantity of merozoites found in the supernatant after egress was not significantly different than with wild-type controls. Based on these observations, we have concluded that CDPK5 is not essential for egress in *C. parvum*. This may be due to a limited role for CDPK5 in egress or the ability of other mechanisms to compensate for loss of CDPK5. Previous studies in *Plasmodium* have concluded that inhibition of some CDPKs did not affect early parasite development but was important later in the life cycle. This is seen mostly in gamete development, ookinete mobility, and other processes in merogony[208,242,243]. If CDPK5 did not affect invasion, development or egress, it still may affect another stage in the life cycle.

There are seven distinct calcium-dependent protein kinases (CDPKs) found within the *Cryptosporidium* genome. While our data demonstrated that CDPK5 was not essential for egress of merozoites from host cells, we cannot conclude that calcium-dependent protein kinases are not necessary for this process. CpCDPK3 is another calcium-dependent protein kinase with a four EF-hand motif and expressed prior to egress, which may indicate a role in egress[211,214]. Previous studies in *Toxoplasma* have demonstrated a role of TgCDPK3 in egress of tachyzoites[184]. *Cryptosporidium* CDPK3 shares sequence homology (14% identity) with this construct, which could indicate a role in egress.

Subtilisin-like Serine Protease in Egress

We have demonstrated that subtilisin-like serine protease in *Cryptosporidium parvum* plays an essential role in egress of merozoites from host cells. Previous studies have identified SUB1 in apicomplexans as responsible for post-translational proteolytic processing of proteins involved with invasion and egress[225]. Others have demonstrated that these subtilases localize to

the apical regions prior to host cell escape, consistent with prepackaging. In *Cryptosporidium*, subtilase1 (CpSUB1) was noted throughout the lifecycle, but only observed at 12 hour time points[225]. In a prior study, CpSUB1 was localized to the anterior part of the parasite, suggesting localization in micronemes or other secretory vesicles[225]. Consistent with this observation, we observed an increase of CpSUB1 expression at 12-20 hours post-infection, which would suggest prepackaging into secretory vesicles. Prior to these results, the mechanisms of CpSUB1 during egress have not been determined. Our results showed a reduction of 90% in the expression level of CpSUB1 without cytotoxic effects and no effect on housekeeping gene expression. After silencing of SUB1, we observed a drastic decrease in merozoites released from host cells compared to wild-type samples. We also confirmed this observation by quantifying intracellular parasite burden in host cells using rtPCR. At 24 hours post-infection there was a drastic increased burden in silenced samples compared to wild-type samples. Interestingly, at 26 hours post-infection, there were low-levels of parasites detected. The corresponding levels of host cells, determined by rtPCR of human 18s, were maintained through the infection. Therefore, it is possible the parasites are lysing at this timepoint, although further experimentation is required to confirm this observation. Additionally, we've also observed that egress was neither premature nor delayed by observing timepoints before and after 24 hours post-infection.

Interestingly, our observations that CpSUB1 plays a major role in egress closely mirrors the mechanism observed in *Plasmodium*. In *P. falciparum, s*ubtilisin-like serine protease (PfSUB1) is secreted into the parasitophorous-vacuolar space prior to egress, where it cleaves and activates other enzymes that eventually lead to degradation of host membrane for parasite egress[205,244]. In *Cryptosporidium*, CpSUB1 plays a role in invasion by cleavage of gp40/15, ligands involved with invasion of host cells[225,226]. However, this study is the first to describe the role of CpSUB1 in egress. Further studies will need to be carried out to determine the substrates

of CpSUB1, and if mobilization of exonemes discharge the protease into the PV lumen as is observed in *Plasmodium*.

cGMP-Dependent Protein Kinase G in Egress

PKG has been identified as an essential trigger for egress in apicomplexans. *Toxoplasma gondii* PKG is a key regulator of tachyzoite egress in coordination with calcium-dependent protein kinases (CDPKs) [174,183,186]. Loss of PKG in *Plasmodium falciparum* has been associated with inhibition of parasite growth and reduction of egress [232]. Based on these observations, we hypothesized that the PKG homologue in *C. parvum* would play a vital role in regulation of merozoite egress and subsequent completion of the life cycle. Our studies have found a high expression of PKG prior to the time of egress in an *in vitro* model of infection. Lastly, silencing of PKG has shown a drastic decrease of merozoite egress during *in vitro* infection.

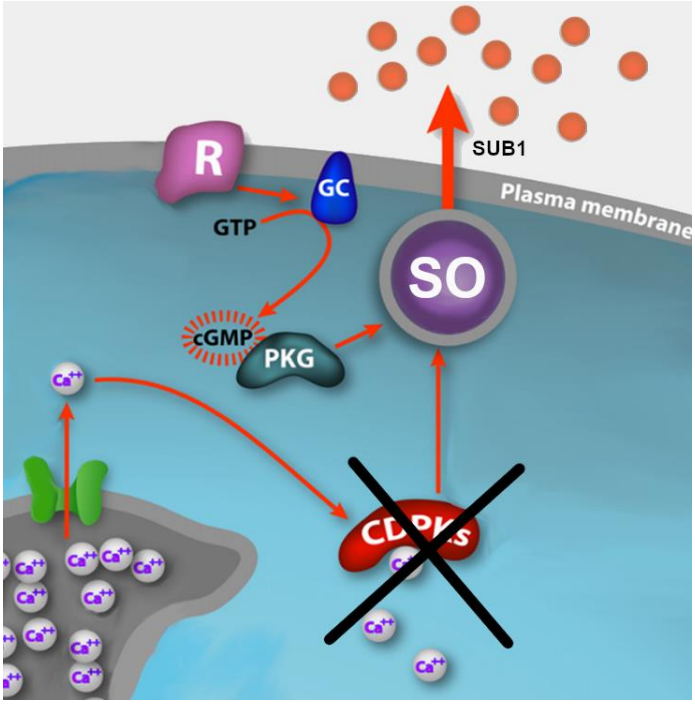


Figure 14. Schematic of *Cryptosporidium* egress of merozoites. Based on the data presented, SUB1 and PKG are essential for egress in *C. parvum*. The relationship between PKG and SUB1 could be causal, but further studies need to confirm this relationship. While CDPK5 was not found to be essential for egress of merozoites, other CDPKs found within the *Cryptosporidium* genome could be further investigated for their role in egress.

Studies in apicomplexans have demonstrated that PKG plays a central role in egress. In *Plasmodium*, PfPKG is found to be control intracellular calcium and, as a consequence, microneme secretion, which has been linked to secretion and activation of PfSUB1[245]. PfPKG is upstream of a signaling cascade involving subtilisin-like serine protease (SUB1) and serine repeat

antigens (SERA), which degrade the parasitophorous vacuolar membrane upon activation [177,179,246]. If the mechanism of egress in *Cryptosporidium* models the process found in *Plasmodium*, these observations would suggest potential substrates for CpSUB1 and CpPKG.

Collectively, the data obtained by this study strongly demonstrates an essential need for SUB1 and PKG in egress of merozoites from epithelial cells *in vitro.* Future studies could focus on determining the relationship between SUB1 and PKG. Previous studies have found a causal relationship between PKG and SUB1, where PKG is thought to initiate the cascade to egress of merozoites in *P. falciparum*[179]. Without the methods to develop mutant *C. parvum* parasites, a conjugated antibody against SUB1 would be useful to observe the migration of secretory organelles prior to egress. Additionally, CDPK5 was not found to be essential for egress of parasites; however other calcium-dependent protein kinases found within the *Cryptosporidium* genome may be crucial for this process (Fig 14). While the data obtained from these studies is compelling, there are a few limitations to our methods. First of which is the use of housekeeping genes to determine the expression profile for SUB1, PKG, and CDPK5. Data normalization is essential to establish reliability of data in real-time quantitative PCR. However, the quality of normalized expression data is limited to the quality of the normalizer gene itself. With metabolic changes associated with parasite replication and nutrient processing, housekeeping genes often fluctuate as the life cycle progresses. Another limitation to this study is the use of RNAi to decrease gene expression. Interference RNA (RNAi) has its disadvantages with incomplete knockdown of genes and experimental variability. In addition, RNAi is easily degraded and limited to observation of gene knockdown during short duration. To develop therapeutics, it may be necessary to investigate genomic modification of SUB1 and PKG such has been described by the Striepen group[247].

## Conclusion

888 Egress is essential step in the life cycle of apicomplexan parasites. Further work needs to
889 be completed to consolidate the observations from egress studies in all apicomplexans. This
890 study is the first to explore the mechanisms of egress in *Cryptosporidium*. Our results
891 demonstrate that SUB1 and PKG are essential components for egress of merozoites from host
892 cells, while CDPK5 is not.

893 Cryptosporidiosis affects millions of people worldwide with suboptimal treatments and no
894 licensed vaccines available. Oral rehydration therapies decrease much of the mortality from acute
895 diarrhea, but effects from persistent and recurring infections such as growth stunting and cognitive
896 impairment are being increasingly recognized[33,34,37,38,53,248]. Recombinant vaccines have proven
897 highly effective at generating antibody responses and preventing infections in which antibody
898 place a critical role[156,249]. However, most evidence suggests minimal to no role of antibody in
899 preventing cryptosporidiosis[91]. A live-attenuated vaccine could potentially be more effective in
900 stimulating the cellular immune response critical in cryptosporidiosis. By decreasing egress,
901 organisms would be limited to a single cycle of infection, which could generate an immune
902 response without causing illness. One option for attenuation would be to knock-out key molecules
903 involved in egress. Development of a vaccine that could prevent establishment of infection in
904 young children would be a major advance in preventing the detrimental effects from infection[138].

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TABLE 1

| Target | ssRNA sequence | Size | Location |
|---|---|---|---|
| SUB1 #1 | GAU GAG CUU GUU GGA AUC AAA UCTG | 25 | 544-569 |
| SUB1 #2 | GAA UGG AUU CUG AAG AAG UAA AAA | 25 | 2837-2862 |
| SUB1 #3 | GTT GAA GGT CGG AGC TCT G | 20 | 106-126 |
| SUB1 #4 | GAA CCA GAT CAG GAA GTT AGG CTT GCA | 26 | 688-714 |
| SUB1 #5 | GAA GAA GAG GAT GAT GGA GA | 20 | 3292-3372 |
| CDPK5 #1 | CAG GGG AGC UUC AGC AAA A | 19 | 218-236 |
| CDPK5 #2 | GGG ACA CAT TTA TGC AGC TAT GTG T | 24 | 183-207 |
| CDPK5 #3 | GAG AGG CTC TTA TGG ATC TGT TGT A | 24 | 576-600 |
| CDPK5 #4 | CAT GCG CTT GTT AAC AGG AAT ATT A | 24 | 517-541 |
| CDPK5 #5 | AGG GAATACAGGA ACAAGGC | 25 | 128-153 |
| PKG | AUA UCU UGU GAA AGA AUA AAG CUA | 18 | 150-175 |

**Table1**. Single-stranded antisense sequences. Antisense sequences sorted by sequence, size and location from 5' end of SUB1, CDPK5, and PKG mRNA, respectively.

TABLE 2

| Primer | Sequence |
|---|---|
| LDH For | GACGCAAGATTGCCGTTATT |
| LDH Rev | TATGTGGAAGCCCAGAAACC |
| SUB1 For | TCAAGTTGTTGCCATATTTATTGGT |
| SBU1 Rev | CCTAAATGTTTCCATGCCATCAA |
| H18s For | CCGATAACGAACGAGACTCTGG |
| H18s Rev | TAGGGTAGGCACACGCTGAGCC |
| PKG Forward | CAACCCCTGAGGTGCATCTAT |
| PKG Reverse | TCGAGAGCTAGAATAATTGAGCCA |
| CDPK5 Forward | TGAATGCGCTAAACGTGTAGG |
| CDPK5 Reverse | ACGAGAAATCTGTTCTTTCCC |

**Table 2**. Primer list for RT-PCR analysis